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14. ABSTRACT In the first year of support we established protocols for efficient in vitro cultivation and infection of mammary gland derived normal, hyperplastic and tumorigenic cells. For the second year we reported the analyses of mammary tumor cells when infected with candidate genes that are likely to promote invasion. We established a three dimensional basement membrane based cell culture system for primary mouse mammary cells that will allow us to examine differences in normal, hyperplasic, tumorigenic and invasive behavior of infected mammary cells in more detail. In the last funding period we studied the effect of inducible oncogenes on established polarized acini. We monitored oncogenic growth, tumor maintenance and cell autonomous tumor regression of primary mammary cells. We managed to identify a special subset of cells, located at the rim of the structures, that was protected from cell death after oncogene withdrawal. This outer layer of cells was capable of re-initiating tumorigenic growth upon renewed doxycycline exposure and may be a surrogate of dormant tumor cells, the potential substrate of tumor relapse.

15. SUBJECT TERMS
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Introduction:
Several hallmarks of carcinogenesis, and of epithelial tumor progression in particular, have been identified during the past few decades. These critical features include uncontrolled proliferation, insensitivity to negative growth regulation, evasion of apoptosis, angiogenesis, and metastasis (1).
To investigate molecular players in breast cancer progression, we combined well characterized mouse breast cancer models expressing the primary oncogenes Neu or Wnt-1 with a tissue-specific viral delivery system (TVA system) that will allow the somatic delivery of secondary lesions. Multiple genes can be introduced and the cooperative action of these genes can be studied to see if they are crucial to promote metastasis.

We employed the established protocols for efficient *in vitro* manipulation and infection of mammary gland-derived normal, hyperplastic and tumorigenic cells, derived in the first funding periods of this grant to address different steps in breast cancer progression. We intended to study the dynamics of tumor initiation as well as the process of tumor regression with time laps and confocal microscopy methods. Since the mouse is not an easily accessible system to perform these analyses, we decided to embark on a relevant *in vitro* approach. We succeeded to set up a three dimensional culture approach that enabled us to monitor primary mammary cells.

Report

During the first funding period we established protocols for efficient *in vitro* cultivation of mammary gland-derived normal, hyperplastic and tumorigenic cells, mainly from transgenic mice programmed to express *Wnt1* or *Neu* oncogenes and the avian virus subgroup A receptor, *TVA*, in the mammary gland. We have been able to infect cultivated cells at different stages of tumor progression with RCAS-A viruses (Fig.1A) and lentivirus (HIV) vectors pseudotyped with avian virus EnvA (Fig. 1B). Since this system produces only low levels of viral proteins, no infectious particles are formed and the virus is unable to spread. This leaves the viral receptor available for re-infection and allows introduction of several candidate genes and the study of cooperative action of these genes to promote tumor growth, invasion, and metastasis. Indeed, *in vitro* infection of TVA positive mammary gland cells with two different populations of viruses carrying GFP and RFP showed a substantial proportion of double positive cells (Fig. 1B).

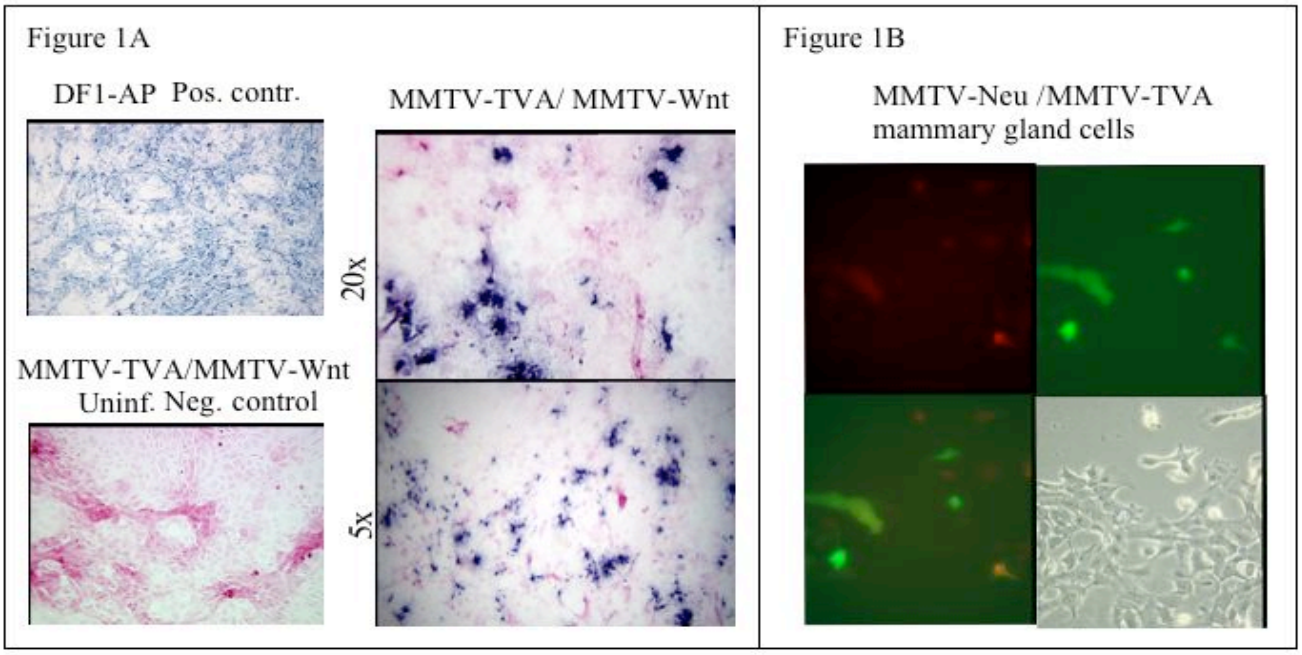

Figure 1A Primary cells expressing TVA under the control of the MMTV promotor can be efficiently infected in cell culture. Infection with RCAS virus carrying thermostable human Alkaline Phosphatase (AP) is shown

Figure 1B Cells derived from MMTV-Neu/MMTV-TVA mammary gland were infected with EnvA pseudotyped lentivirus that carries GFP and with RCAS-RFP virus at the same time. FACS analyses verified that more than 50% of RFP positive cells express GFP as well.

In a first attempt to identify genes that can augment tumorigenesis in this model system, we cloned different genes that had been suggested to promote late stage tumor progression and metastasis, including *TGF-beta1*, *Snail*, *Twist*, *amphiregulin*, and *CXCR4* (2-9) into RCAS and lentiviral vectors pseudotyped for EnvA. All of the RCAS viruses grow efficiently in DF1 cells, producing stocks that were concentrated to titers of 10^8 infectious particles per ml.

Viruses carrying different genes elicited different behaviors in mammary tumor cells on collagen coated plates. Figure 1 shows an example of cultivated *MMTV-TVA/MMTV-Neu* tumor cells, infected with RCAS viruses carrying markers and candidate progression factors. Cells infected with RCAS-*AP* or RCAS-*GFP* grow in a polarized manner, resembling cells in tumors from *MMTV-Neu* mice (Fig.2A; 30-40% GFP positive cells as estimated with fluorescence microscope), cells infected with *RCAS-Twist* show unpolarized patches (Fig. 2B); while cells infected with viruses encoding *Twist*, *Snail* and *Tgf-beta1* display more severe aberrant phenotypes, including single fibroblastoid looking cells (Fig. 2C).

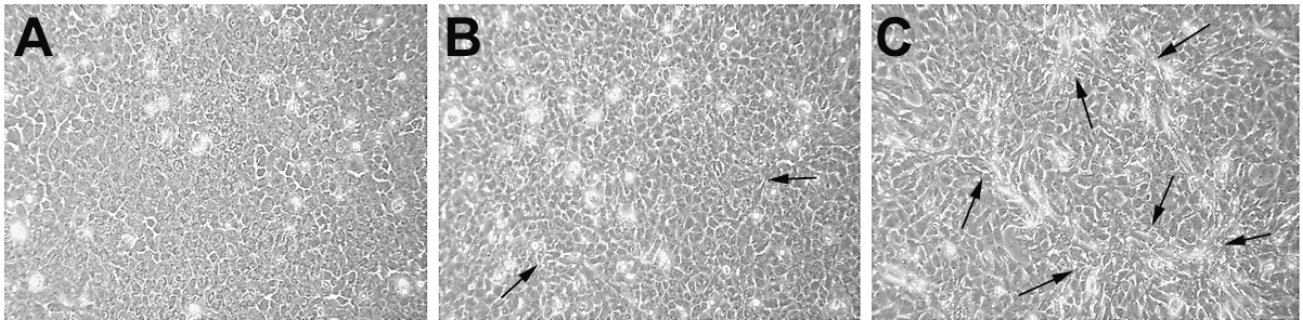

Figure 2 Growth phenotypes of infected *MMTV-Neu/MMTV-TVA* tumor cells. Tumor cells infected with RCAS viruses carrying *AP* and *GFP* (**A**); RCAS-*Twist* **(B)**; RCAS viruses carrying *Twist*, *Snail* and *Tgfb1* (**C**). arrows indicate depolarized patches

Following infections of TVA-positive mammary tumor cells from different mouse strains (*MMTV-Neu* and *MMTV-Wnt1* transgenics), we are currently assessing efficiency of tumor formation and enhanced metastasis to the lung after fat pad transplantation. In initial experiments, we placed cells infected with one of the above mentioned tumor progression factors or GFP/alkaline phosphatase AP alone in cleared fat pads (Rag-/- mice). We let tumors grow until we had to sacrifice animals according to RARC guidelines (2 to 3 months) and then surveyed lungs and other organs for metastases. All cultured, infected and replanted primary tumor cell populations were able to form tumors with similar latency and some displayed visible metastases at the time of sacrifice. To our surprise also cells that did only harbor control genes (GFP or AP) would metastazise with comparable efficiency, questioning the validity of our approach.

Given the multitude of parameters and difficulties of the *in vivo* metastases experiments, we started to assay growth patterns and invasive behavior in three dimensional basement membrane extract gel assays.
At the end of the second year and mainly in the final year of funding we established conditions for cultivation of mammary gland-derived normal, hyperplastic, and tumorigenic cells in three dimensional, basement membrane-extract gels. Experiments with epithelial cells grown in two dimensional cultures have severe limitations with respect to polarized morphology, specialized cell–cell contacts, and attachment to an underlying basement membrane. All of these features are necessary for the proper control of cellular proliferation, survival, differentiation and secretion (10). Cultivation of epithelial cells on a 2D planar surface impairs epithelial polarization, since nutrients and growth factors in the culture medium cannot pass the tight junctions of a fully polarized monolayer and fail to reach their basolaterally located receptors facing the plastic surface. These problems can be circumvented by culturing cells in three-dimensional (3D) collagen gel cultures, in which fully polarized cells form organotypic structures. These culture systems are able to recapitulate essential structural features of glandular epithelium and are amenable to experimental manipulation and

detailed microscopic analyses. Furthermore, gels can be fixed, cut, and processed for immunohistochemisty and immunofluorescence.

Efforts to develop 3D-culture systems to study neoplasia have generally utilized immortalized cell lines (6, 11-16).
These organotypic culture models have been instrumental in uncovering biochemical mechanisms and cell biological behaviors believed to be responsible for the early stages of mammary tumor development (10, 17, 18). However, the mutational status of immortal cell lines derived from long term culture efforts remains elusive (19, 20). We believe studying oncogene cooperation in a primary cell derived organotypic model to be a more valid strategy of understanding the phenotypes observed in the transgenic animals. In fact, primary cells have been successfully grown on a reconstituted basement membrane matrix to form hollow acini that were sealed by tight junctions (21).

We successfully established embedded 3D culture conditions for both cells derived from normal mammary tissue and mammary tumor cells. Untransformed primary mammary cells grow in hollow acinar structures, while tumorigenic cells form enlarged solid balls (Fig. 3). We also used RCAS virus vector carrying GFP in order to test, whether infected tumor cells can still be grown in the 3D conditions and obtained solid green structures in part of the gels, opening the possibility to use our delivery system for these experiments.

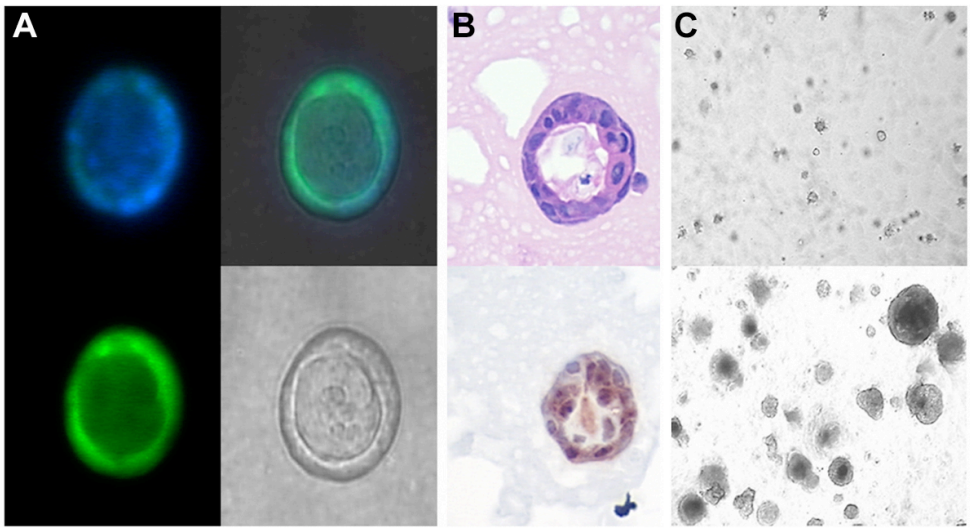

Figure 3. Organotypic growth of normal and tumorigenic primary mammary cells in 3D culture conditions **A**. Mammary gland cells harvested from a *beta-actin-GFP* transgenic mouse are forming acinar structures. Nuclei are stained with Hoechst 33342 (upper left); GFP expression can be observed in the gel (lower left); brightfield (lower right) and overlay (upper right) are shown. **B.** 3D gels can be cut and stained for H&E (upper panel) and TVA (lower panel). **C.** Comparison of structures formed by normal mammary cells (small acini; upper panel) and Wnt1-expressing breast tumor cells (large solid structures; lower panel); both pictures taken under 2.5x magnification.

Next we manipulated solid structures obtained from single tumor cells with soluble, activated TGFbeta to test invasive behavior in the three dimensional basement membrane extract gels. We observed a growth inhibitory effect on a large proportion of cells in the treated gels, which was not too surprising, since TGFbeta1 is a potent growth inhibitor, with tumor-suppressing activity. However, cancers are often refractile to this growth inhibition either because of genetic loss of TGF-beta signaling components or, more commonly, because of downstream perturbation of the signaling pathway, such as by Ras activation (22). Indeed, a subpopulation of Wnt1 tumor cells formed elongated, presumably non polarized, invading structures (Figure 4B) that could not be seen in untreated control gels

(excluding the possibility of remaining contaminating fibroblasts). Other surviving structures displayed cells on the surface that seem to sprout into the 3D matrix (Figure 4C).

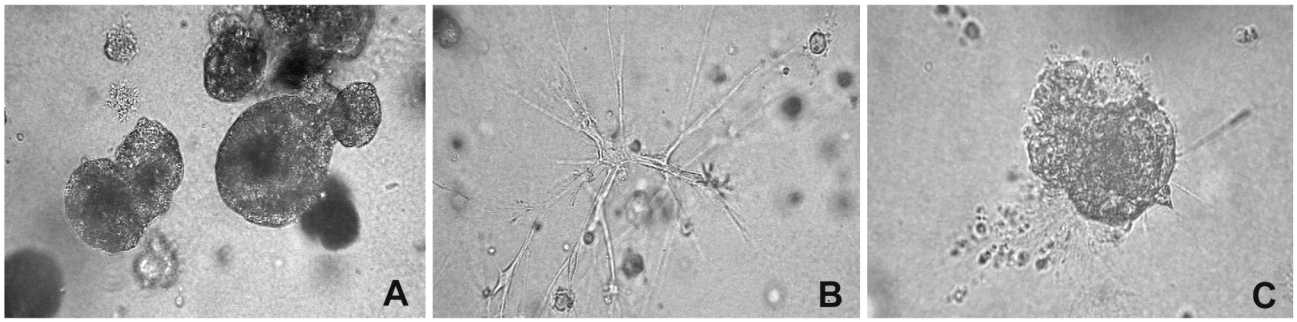

Figure 4. Growth of Wnt1 expressing tumorigenic primary mammary cells -treated or left untreated with TGFbeta- in 3D culture conditions **A**. Wnt1-expressing breast tumor cells grew into smooth surfaced, solid balls. 20x magnification **B.** When treated with 5 ng/ml TGFbeta for 10 days (starting 2 days after seeding), a subset of Wnt1-expressing breast tumor cells formed interconnected, elongated invasive structures. 20x magnification **C.** Another subset of seeded Wnt1-expressing breast tumor cells grew more solid structures. In contrast to untreated control structures, these comparably smaller solid structures displayed rough edges that showed single sprouting cells. 40x magnification

There have been successful efforts to study mechanisms of tumor emergence, regression and recurrence using transgenic mice that bear tetracycline regulated oncogenes (23-25). These animals allow de-induction of the tumor initiating oncogene in order to mimic an idealized targeted drug treatment. We recently described a tri-transgenic *TetO-MYC;TetO-Kras^{D12};MMTV-rtTA (TOM;TOR;MTB)* line that coordinately expresses *MYC* and mutant *Kras* oncogenes in mammary epithelial cells in animals receiving doxycycline in the food (25). The cooperation of these powerful oncogenes induced palpable mammary tumors, that regressed completely after weaning mice from doxycycline. Surprisingly, although we observe total shutdown of initiating oncogenes, recurrent tumors developed in the majority of animals.

To clarify, how a polarized terminal end bud can, in the course of tumor emergence and complete remission, produce a subset of specialized cells that manage to survive the oncogene withdrawal, we intended to study the dynamics of tumor initiation as well as the process of tumor regression with time lapse and confocal microscopy methods. Since the mouse is not an easily accessible system to perform these analyses, we decided to utilize our 3D system for this analyses. We seeded the gels with mammary cells derived from trigenic *TetO-Myc/TetO-Kras^{G12D}/MMTVrtTA* mice, that allow regulation of Myc and Kras G12D oncogenes by administration of doxycycline. As expected, uninduced cells form hollow polarized acinar structures, similar to structures formed by cells derived from wild type animals (Fig.5A,D). When induced in culture with doxycycline (1mg/ml) to overexpress Myc and mutant Kras, these structures grew into solid structures within 4 days (Fig.5B,E). A closer look at acini behavior upon doxycycline treatment, employing time lapse microscopy, showed that acini were filling in first before expanding in size, reminiscent of ductal carcinoma in situ (DCIS). At the same time the dynamically growing structure lost the organized epithelial polarization pattern of the un-induced acinus.
When we elute doxycycline from the gels by multiple washes with media, growth ceases and the inner region of the solid clusters of cells clears, in a Caspase 3 dependent manner.

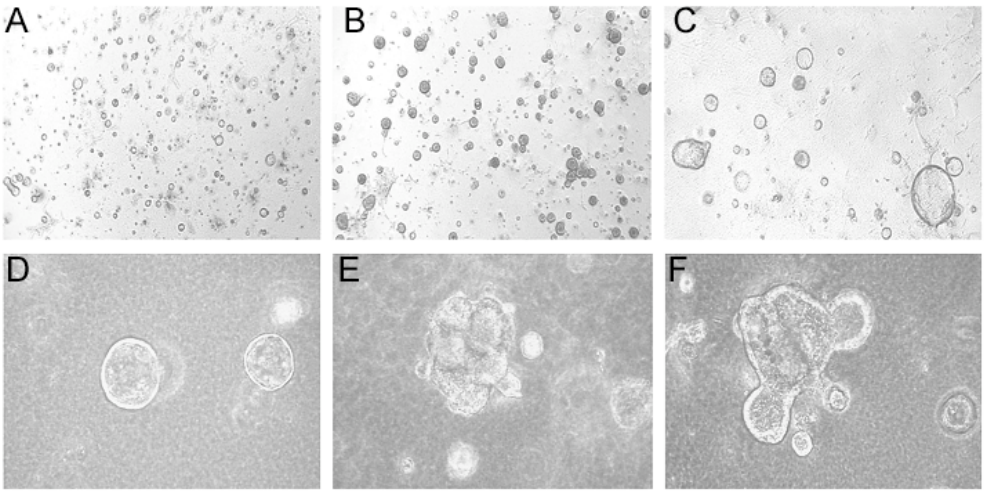

Figure 5. Regulation of transformation in primary mammary cells with inducible Myc and mutant Kras
Appearance of primary 3D cultures from previously uninduced trigenic animals before transgenes induction (**A**,**D**), after 4 days of induction in culture (**B**,**E**), and 4 days after doxycycline removal (**C**,**F**); **A**, **B**, **C** 2.5x; **D**, **E**, **F** 20x

We observed that the outermost layer of cells of the spheres remains viable following oncogene de-induction, while re-establishing a polarized monolayer but retaining the irregular shape assumed during doxycycline-stimulation. (Fig.5 C,F). These cells display a phenotype consistent with progenitors of mammary epithelium: they exclude Hoechst dye 33342, and –when harvested and disaggregated-- reform acini in 3D cultures and repopulate mammary fat pads more efficiently than cells harvested from uninduced acini. Furthermore, secondary acini redevelop into solid spheres after re-exposure to doxycycline, indicating that the residual cells retain responsiveness to the original oncogenes and may represent the type of cells that give rise to recurrent tumors. Our ability to recover such cells from the 3D cultures should now permit a much more extensive characterization of the properties and fates of the surviving cells. In general, we have now a system in hand that allows infection, growth of cells in an organotypic fashion *in vitro* and testing of recovered cells in a cleared fat pad *in vivo*.

To enlarge the cohort of factors to be tested in the experiments outlined above, we are guided by published gene expression profiling studies that have identified upregulated genes that may play a role in during initiation of tumorigenic growth and metastasis.
To help us choose among the many candidate genes described in these several studies, I am working with the Computational Biology Center run by Dr. Chris Sander at MSKCC to narrow down the most promising candidates. Using the INGENUITY tool we were able to identify first interesting clusters of genes that might be important to drive cells towards metastasis. One such network is displayed in Figure 6. We highlight two genes, endoglin and disabled-2 (DAB-2), which associate with the TGFbeta receptor complex –a crucial signaling complex in EMT and metastasis-- and modify the intracellular response to TGFbeta ligands. Endoglin (CD105) is required during embryonic angiogenesis, selectively binds TGFbeta1/3 and can modulate TGFbeta signaling by diverting signaling to different receptor complexes. Endoglin-/- mice have a phenotype strikingly similar to TGF1-/- mice. Furthermore, endoglin is the only gene in the TGFR signaling pathway, the expression of which correlates with bad prognosis in breast cancer patients(26). DAB2 is required for efficient phosphorylation of SMAD proteins by the type I receptor, interacts with Grb2 and thus also provides a link of TGFR signaling to the Ras/MapK pathway.

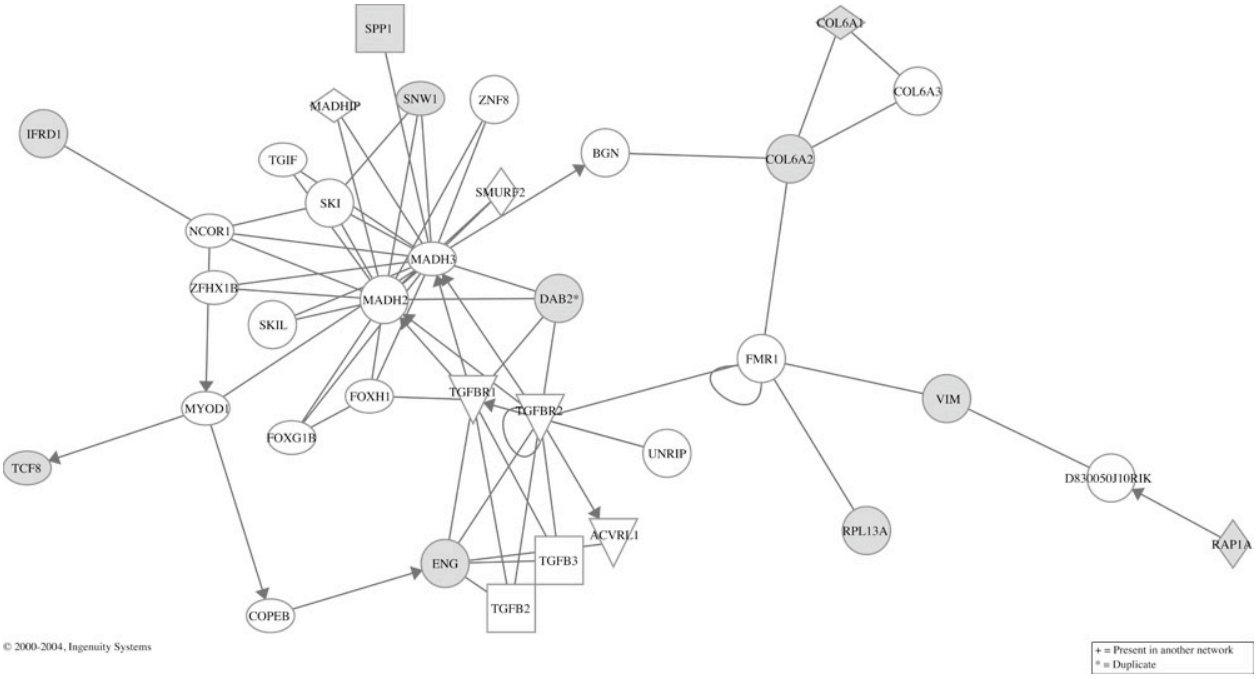

Figure 6. Signaling network identified when the gene expression profiles of metastatic cell lines were compared and an overlapping cluster of genes was submitted for analyses in INGENUETY. Description of the two genes closest related to the TGFbeta complex see main text.

We are further pursuing our efforts to identify genes involved in breast cancer tumor progression, by performing affimetrix analyses on spheres at different stages of doxycycline induction and regression after removal of doxycycline. Signatures will be analyzed accordingly, and genes identified as candidates for breast cancer progression tested in our novel combined *in vitro/in vivo* approach.

Key Research Accomplishments

.) Mammary tumor cells infected with genes that have been proven to promote late stage tumor progression and metastasis (TGFβ, snail, twist) show different morphologies when grown on collagen1 as compared to GFP/AP infected cells.
.) We established conditions for cultivation of mammary gland-derived normal, hyperplastic, and tumorigenic cells in three dimensional, basement membrane-extract gels.
.) We are able to manipulate solid structures obtained from single tumor cells with soluble, activated TGFbeta to see invasive behavior in the three dimensional basement membrane extract gels.
.) We can grow mammary cells derived from tri-transgenic *TetO-Myc/TetO-Kras^{G12D}/MMTVrtTA* mice, that allow regulation of Myc and Kras G12D oncogenes by administration of doxycycline, in the three dimensional basement membrane extract gels.
.) Surviving cells after oncogene withdrawal display a phenotype consistent with progenitors of mammary epithelium: they exclude Hoechst dye 33342, and –when harvested and disaggregated-- reform acini in 3D cultures and repopulate mammary fat pads more efficiently than cells harvested from uninduced acini.

Reportable Outcomes

Podsypanina K, Du YC, Jechlinger M, Beverly LJ, Hambardzumyan D, Varmus H (2008) Seeding and propagation of untransformed mouse mammary cells in the lung. *Science* **321:** 1841-1844

MODELING ONCOGENE DEPENDENCE IN THREE-DIMENSIONAL CULTURES OF PRIMARY MOUSE MAMMARY CELLS by Martin Jechlinger, Katrina Podsypanina and Harold Varmus in submission at Nature Cell Biology

Poster-presentation at the LXX Cold Spring Harbor Symposium “Molecular Approaches to Controlling Cancer”:
IDENTIFICATION OF MECHANISMS OF BREAST CANCER METASTASIS USING TISSUE SPECIFIC VIRUS DELIVERY
Martin Jechlinger and Harold E. Varmus

Poster presentatin at the Nature-CNIO conference “Oncogenes and Human Cancer” 10/2007:
MIMICKING ONCOGENE-DEPENDENT TUMORIGENESIS IN AN IN VITRO, THREE DIMENSIONAL COLONY ASSAY
Marin Jechlinger, Katrina Podsypanina and Harold E.Varmus

Poster presentatin at the DOD Breast Cancer Research Meeting “Era of Hope” 06/2008
MIMICKING ONCOGENE-DEPENDENT TUMORIGENESIS IN AN IN VITRO, THREE DIMENSIONAL COLONY ASSAY P37-16
Marin Jechlinger, Katrina Podsypanina and Harold E.Varmus

Conclusions

.) Given the multitude of parameters and difficulties of the *in vivo* metastases experiments, we started to assay growth patterns and invasive behavior in three dimensional basement membrane extract gel assays.
.) We established a three dimensional basement membrane based cell culture system for primary mouse mammary cells that will allow us to examine differences in normal, hyperplasic, tumorigenic and invasive behavior of infected mammary cells in more detail.
.) Using primary cells that carry regulate able oncogenes, we studied mechanisms of oncogenic growth, tumor maintenance and cell autonomous tumor regression.
.) We managed to identify a special subset of cells, located at the rim of the structures, that was protected from cell death after oncogene withdrawal.
.) This outer layer of cells was capable of re-initiating tumorigenic growth upon renewed doxycycline exposure and may be a surrogate of dormant tumor cells.

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